Mineralisation of ^{14}C-phenanthrene in PAH-diesel contaminated soil: Impact of *Sorghum bicolor* and *Medicago sativa* mono- or mixed culture

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Abstract

Plant-assisted biodegradation can offer a cost-effective and sustainable approach for the bioremediation of PAHs in soil. As such, selecting the most appropriate plant species is important. The potential for plant-assisted biodegradation of complex PAH-diesel mixtures in soil by sorghum (*Sorghum bicolor*) and alfalfa (*Medicago sativa*) grown as monocultures and mixed cultures using ^{14}C-contaminants has not been widely reported. The objective of this study was to assess ^{14}C-phenanthrene mineralisation profiles in mixtures of PAH-diesel in soil in the presence of *Sorghum bicolor* and *Medicago sativa.* Soil was spiked with PAHs and diesel, after which *M. sativa* and *S. bicolor* were introduced and grown as mono- or mixed-cultures. The toxicity of the PAH-diesel oil mixture in the planted treatments, as well as its effect on the mineralisation of ^{14}C-phenanthrene were evaluated. Monocultures of both plant species tolerated the complex PAH-diesel mixtures based on growth and survival, and increased rates and extents of ^{14}C-phenanthrene mineralisation in soil. The influence of PAH concentration on ^{14}C-phenanthrene mineralisation profiles varied in planted and unplanted treatments. The rates and extents of ^{14}C-phenanthrene mineralisation tended to decrease in diesel amended soil, especially at low PAH concentrations. To the best of the authors' knowledge, this is the first report of ^{14}C-phenanthrene mineralisation patterns in complex PAH-diesel oil mixtures contaminated soil especially with respect to the specified plant species. The findings offer new insights on mono- and multi-species phytotoxicity as well as plant-assisted biodegradation of PAH mixtures in soil which may be useful in the risk assessment and remediation of contaminated sites.

Keywords: PAH mixtures; diesel oil amendment; Phytotoxicity; *Sorghum bicolor*; *Medicago sativa*; ^{14}C-phenanthrene mineralisation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are hydrophobic organic contaminants with two or more fused benzene rings together. Generally, these compounds are of concern to human and environmental health due to their carcinogenicity, toxicity, and persistence in the environment (Juhasz and Naidu, 2000). The USEPA has classified 16 PAHs as priority pollutants including phenanthrene (Phe), benzo[a]anthracene (BaA), and benzo[a]pyrene (BaP) (USEPA, 2008). Although PAHs are released into the environment from natural combustion of organic matter, anthropogenic activities constitute the most important sources (Wilson and Jones, 1993). For example, burning of fossil fuel, coal, and wood, vehicular emissions, heating, and accidental spills of crude oil and other petroleum products among others are well known sources of PAH release into the environment.

Soil is considered a major sink for PAHs in the environment (Wild and Jones, 1995; Semple *et al.*, 2001). PAHs can be found as complex mixtures in soil, where they associate with other chemicals such as phenols, aliphatic hydrocarbons and metals (Allan *et al.*, 2007; Thavamani *et al.*, 2012). PAHs also exist in co-contamination with non-aqueous phase liquids (NAPLs) such as transformer oil from electrical cables and diesel oil from deliberate and accidental oil spillage around petroleum hydrocarbon contaminated sites (Molina-Barahona *et al.*, 2004). The implication is that co-contamination is likely to change the fate and behaviour of PAHs in soil (Lee *et al.*, 2003; Couling *et al.*, 2010). This effect has been previously observed under a range of conditions by different authors such as Swindell and Reid (2006) or Towell *et al.* (2011).

Considering the environmental implications of the presence of these contaminants in soil, various studies have reported the potential of plant-assisted biodegradation of PAHs in soil (Banks *et al.*, 2003; Meng *et al.*, 2011; Chen *et al.*, 2016; Deng and Zeng, 2017). Although the mechanisms promoting plant assisted biodegradation of PAHs and other hydrophobic organic contaminants are not fully understood, different processes have been observed to affect the biodegradation process (Oyelami *et al.*, 2013). Among these, plant identity (Panchenko *et al.*, 2016) and root exudates have been hypothesised to play an important role (Fan *et al.*, 2008;

Wenzel, 2009; Gao *et al.*, 2017). Mixed cultures of two or more plant species can enhance rates and extents of biodegradation (Chen *et al.*, 2016), potentially due to nutrient- and metabolites-richer rhizosphere, when compared to their corresponding monocultures (Wenzel, 2009). Since the effectiveness of plant-assisted biodegradation may differ with plant species (D'Orazio *et al.*, 2013), finding appropriate plant species mix may represent a confounding factor for phytoremediation (Panchenko *et al.*, 2016; Thijs *et al.*, 2017).

Plant-assisted biodegradation of complex PAH-diesel oil mixtures in soil, measured through a ^{14}C-PAH mineralisation approach, in the presence of mono- or mixed- cultures of *Medicago sativa* L. (Fabaceae) and *Sorghum bicolor* (L.) Moench (Poaceae) has not been previously reported. Plant-assisted biodegradation in this present study was used to imply increased microbial mineralisation of ^{14}C-phenanthrene, or microbial activities, in planted soils when compared to corresponding unplanted controls. For this study, it was hypothesised that (i) both *M. sativa* and *S. bicolor* would show tolerance in PAH-diesel oil mixture contaminated soil, regardless of PAH concentration or diesel amendment; (ii) Increases in PAH mixture concentration, and diesel amendment, would decrease rates and extents of ^{14}C-phenanthrene mineralisation in soil; (iii) rates and extents of ^{14}C-phenanthrene mineralisation would be greater in planted treatments (monocultures or mixed cultures), and (iv) rates and extents of ^{14}C-phenanthrene mineralisation in treatments associated with mixed cultures would be greater than those of monocultures. To address these hypotheses, the following objectives were set: (i) to assess the tolerance (growth and survival) of *M. sativa* and *S. bicolor* in PAH-diesel oil mixture contaminated soil; (ii) to assess microbial mineralisation of ^{14}C-phenanthrene in soil spiked with a mixture of three PAHs and amended with diesel oil, and (iii) to evaluate and compare microbial mineralisation of ^{14}C-phenanthrene in PAH-diesel oil mixture in planted and unplanted treatments.

2. Materials and Methods

2.1. Chemicals and other materials

Non-labelled phenanthrene (>98%), benzo[a]pyrene (>97%), benzo[a]anthracene (>95%), sodium hydroxide (reagent grade), plate count agar (Fluka analytical), and toluene were purchased from Sigma-Aldrich, UK. [9-^{14}C] phenanthrene (3.7 MBq/ml) was obtained from American Radiolabeled chemicals, Inc., USA, Goldstar liquid scintillation cocktail (LSC) from Meridian, UK, general purpose agar (agar-agar), general purpose grade Ringer's solution tablets, acetone (HPLC grade), as well as the chemicals used for preparing minimum basal salts (MBS) solution were acquired from Fisher Scientific, UK. Seeds of *M. sativa* and *S. bicolor* were purchased from Moles Seeds Ltd., UK and Chiltern Seeds, UK respectively. Commercial diesel was obtained from a local UK petrol station.

2.2. Soil preparation

A pristine agricultural soil was collected from a depth of 5 – 20 cm, from Myerscough Agricultural College, Preston, Lancashire, PR3 0RY, UK. The soil was a clay-loam (Dystric Cambisol) (FAO, 1988). Soil was air-dried and then passed through a 2 mm sieve. Thereafter, the sieved soil was stored in the dark at 4 ^{o}C until needed. Soil properties have been previously determined (Couling *et al.*, 2010) and are presented in Table SI 1. Air-dried soil was spiked with ^{12}C-PAH standard (ΣPAH = Phe + BaP + BaA) at 100 mg kg^{-1} and 300 mg kg^{-1}, as well as diesel (0.1% w/w) when applicable. Spiking was done using an inoculum approach following the protocol described by Doick et al. (2003). Briefly, the soil was rehydrated to approximately 35% moisture content with deionised water, after which 3 batches of 250 g soil were placed in a mixing bowl and spiked with ^{12}C-PAH standard in acetone:toluene (1:1, v/v) carrier solvent mixture. Solvent was allowed to disperse in soil and vented off in a fume cupboard. Soil was then thoroughly homogenised and distributed in pots according to the treatments described in Table 1.

2.3. Plant-assisted biodegradation test

2.3.1. Assessment of seedling emergence and phytotoxicity

Seedling emergence and growth test of both plant species was conducted following relevant OECD and USEPA guidelines (OECD., 2006; US EPA, 2012) with slight modifications. Prior to the growth test, a seed viability test was conducted using seeds (*n* = 10) of each species placed on a moistened filter paper in a petri dish. The petri dish was covered and placed in a controlled temperature room (21 ± 1 ^{o}C) and assessed daily for germination. The pot experiment was conducted in a glasshouse and had a completely randomised block design with three replicates. The specific treatments are described in Table 1. Plastic pots (90 mm) were filled with 50 g soil, with a disc of filter paper fitted at the bottom to avoid soil loss. In addition, individual pot trays were fitted under each pot to control any leachate and avoid cross contamination. For monocultures, 10 seeds were sown into the pots, whereas 5 seeds each were sown for the mixed cultures (i.e. *M. sativa* + *S. bicolor*). . Germination, survival and general visual detrimental effects were assessed daily, while percentage seedling emergence and growth was determined after 21 d. Further, weekly measurements of plant heights were made while other visual toxic effects were also observed. At the end of the growth assay, planted treatments were destructively sampled in order to determine plant biomass. The shoots were harvested from the soil surface while the roots were carefully harvested after inverting the pots on a clean polythene sheet. Afterwards, roots were gently rinsed to detach soil from the surface and then dried with a clean paper towel. The fresh weights of the shoots and roots were measured, after which they were oven-dried at 60 ^{o}C for 24 h and their dry weights assessed. The root/shoot biomass ratios were then calculated.

2.3.2. ^{14}C-Respirometry assay

To assess plant-assisted biodegradation, evolution of ^{14}C-phenanthrene mineralisation in planted (after 0 and 21 d) and unplanted soils (after 0, 21, and 42 d) was monitored in 250 ml modified Schott bottles (*n* = 3) at 20 ± 1 ^{o}C, following the methods described by Reid et al. (2001). The biodegradation parameters assessed in this study include (i) the lag phase (defined as the time taken to reach 5% mineralisation); (ii) the fastest rate (%$^{14}CO_2$ d^{-1}), and

(iii) the cumulative extent of mineralisation expressed as a percentage of the initial ^{14}C-phenanthrene, which has been mineralised to $^{14}CO_2$ during each sampling time.

2.3.3. Enumeration of microbial cell numbers

The number of indigenous microbes (total heterotrophs and PAH degraders) assessed as colony-forming units per grams soil dry weight (CFUs g^{-1}) was estimated after 0 and 21 d (planted treatments), and 0, 21, and 42 d (unplanted treatments) following standard aseptic plate counting techniques (Lorch *et al.*, 1995). Cultures were grown (10 days, 25 ± 1 ^{o}C). Microbial colonies were assessed after 4, 7 and 10 d. The ratio of degraders to total heterotrophs was also determined.

2.3.4. Statistical analysis

Data analysis was carried out using Sigmastat 13.0 (Systat Software, Inc.), and graphs were presented using SPSS Statistics (IBM Corp, version 24) and SigmaPlot for Windows 13.0 (Systat Software, Inc.). The level of significance was at $p < 0.05$. Shapiro-Wilk Test - was used to determine normality of data whereas Levene's Test was used to determine equality of variance between groups. Statistical differences between groups were tested using one-way ANOVA ($p < 0.05$). When $p < 0.05$, Tukey's Post Hoc was used to identify the locations of differences between groups. Where Levene's Test fails ($p < 0.05$), Games Howell's Test which assumes variance non-equality was used for Post-Hoc analysis.

3. Results and Discussion

3.1. Effects of PAHs and diesel on seedling emergence and growth

After 21 d incubation following sowing, no significant differences were observed regarding the percentage of emergence ($p > 0.05$), even though values measured in soil spiked with 100 mg kg^{-1} ΣPAH were consistently greater than in soil amended with 300 mg kg^{-1} ΣPAH (Table SI 2). Plant heights also followed this trend ($p > 0.05$) in both mono- and mixed-cultures when compared to the control (Figure 1). Plant tolerance in PAH contaminated soils has been previously reported (Banks *et al.*, 2003; Cheema *et al.*, 2010; Hamdi *et al.*, 2012). For instance, the heights of *M. sativa*, *Brassica napus,* and *Lolium* sp. in pyrene amended soil were statistically similar to the uncontaminated controls which may imply species tolerance in the contaminated soil used (Ghanem *et al.*, 2010). However, PAHs in soil are generally not acutely toxic to plants (Chouychai *et al.*, 2007; Sverdrup *et al.*, 2007; Khan *et al.*, 2012). PAHs may be unavailable to interact with plants due to sorption in soil, a phenomenon which increases with increasing PAH hydrophobicity as well as soil organic matter content (Luthy *et al.*, 1997), and may thereby minimise PAH toxicity to plants in soil. Some studies however reported that diesel oil affected the germination and seedling emergence of plants and this effect has been attributed to volatile constituents of diesel fuel (Adam and Duncan, 2002; Bamgbose and Anderson, 2015). However, these plant growth effects are reduced significantly with ageing (Bona *et al.*, 2011; Wei *et al.*, 2017). Both *S. bicolor* and *M. sativa* tolerated the complex PAH-diesel oil mixtures contaminated soil as regards seedling emergence and plant growth under the prevalent assay conditions and no apparent signs of stress were observed. Such tolerance might be attributed to a combination of plant morphological and physiological characteristics, and soil-PAH interactions (Wenzel, 2009; Hamdi *et al.*, 2012; de Boer and Wagelmans, 2016).

3.2. Effects of PAHs and diesel on plant biomass

A change in plant root biomass is also an important parameter that can be monitored during plant-enhanced biodegradation (Cheema *et al.*, 2010). High plant root biomass may favour microbial activity in soil through enrichment of rhizosphere (Banks *et al.*, 2003; Fan *et al.*,

177 2008; Wenzel, 2009). With respect to varying PAH concentrations and diesel amendment,
178 variations in plant biomass among treatments were consistently observed in this study (Table
179 2). Root biomass (dry weight) of *S. bicolor* across all treatments was greater than in the control.
180 The greatest biomass value of *S. bicolor* was recorded in soil spiked with 100 mg kg^{-1} ΣPAH
181 and amended with diesel, as it exceeded the control by approximately 2 fold ($p < 0.05$).
182 However, *M. sativa* exhibited a significantly greater ($p < 0.05$) biomass only in soil spiked with
183 100 mg kg^{-1} ΣPAH and amended with diesel, compared to the control. This putative hormetic
184 response has been previously reported for *M. sativa,* where the plant roots were stimulated in
185 soils contaminated with 1% and 1.5% of petroleum hydrocarbons, compared to controls (Kirk
186 et al. 2002), including in corn in crude-oil contaminated soil (Salanitro et al. 1997). Observation
187 of treatments with the same ΣPAH concentration (i.e. either 100 mg kg^{-1} or 300 mg kg^{-1}) in this
188 study revealed that root biomass and root/shoot biomass ratio were generally greater in the
189 diesel amended than unamended soils for both mono- and mixed-cultures (Table 2). For
190 example, root biomass of *S. bicolor* and *M. sativa* monocultures in soil spiked with 100 mg kg^{-1}
191 ΣPAH and amended with diesel was greater by 33% and 31% respectively, compared to
192 similar treatments without diesel amendment. In the same regard, root/shoot biomass ratios
193 was greater by 40% and 18% in the 100 mg kg^{-1} treatments with diesel for *S. bicolor* and *M.*
194 *sativa* respectively*,* as well as by 28% and 45% in the 300 mg kg^{-1} treatments with diesel.
195 Generally, diesel amendment appeared to inhibit the adverse effects of PAHs on plant
196 biomass and root/shoot biomass ratios; this was one of the key observations in this study. It
197 is suggested that diesel in diesel-amended treatments may have promoted PAH partitioning
198 into the diesel phase (Boyd and Sun, 1990), especially at low PAH concentrations in soil. This
199 is such that closely-associating roots in the diesel-amended soil show minimal effects on
200 biomass production compared to diesel-unamended treatments.
201 Overall, soil spiked with 100 mg kg^{-1} and amended with diesel showed the greatest root
202 biomass and root/shoot biomass ratios for both species within all treatments and growing
203 patterns. With increase in ΣPAH concentration from 100 mg kg^{-1} to 300 mg kg^{-1}, root biomass
204 and root/shoot biomass ratios generally decreased, especially for *M. sativa*; however, the

differences were not statistically significant ($p > 0.05$). These results are similar to those presented by Cheema et al. (2010). The authors reported that after 65 d of plant growth, root biomass and root/shoot biomass ratio of *M. sativa* were mostly affected in soil amended with a mixture of 200 mg kg^{-1} phenanthrene and 199.3 mg kg^{-1} pyrene, when compared to rape seed exposed to the same treatment. These trends have also been observed for *Zea mays* L., *Lolium perenne* L. and *Trifolium repens*, exhibiting decreased biomass values with increasing concentrations of phenanthrene and pyrene mixtures in loam soil, but the differences were not statistically significant (Xu *et al.*, 2006). These trends may have resulted from inherent non-acute toxicity of PAHs especially at higher concentrations in spiked soils (Wei *et al.*, 2017). In addition, PAH-contaminated soils may inhibit flow of water and nutrients to plants, thereby affecting plant's ability to increase biomass especially at higher PAH concentrations (Reilley *et al.*, 1996). The relationships between root and shoot biomass, especially root/shoot biomass ratios, are important indicators of plant health, although interpretation of such relationships is not always clear-cut (Mokany *et al.*, 2006). Plant root systems utilise water and mineral nutrients from soil, and transports them to plant shoots, while shoot systems fix CO_2 needed for physiological purposes. It is thought that a reduced root/shoot biomass ratio is unfavourable for plants as it indicates shoot proliferation at the expense of root;, however, reduced root/shoot biomass ratio especially at higher concentrations (300 mg kg^{-1} ΣPAH) does not still exclude plant tolerance within the growth assay conditions (Harris, 1992; Cheema *et al*; 2010). One reason for the reduced root/shoot biomass ratios, especially at higher concentrations may have been due to increased root proliferation to allow increased transport of water and nutrients aboveground thereby increasing shoot biomass at the expense of root biomass, and hence a reduced root/shoot biomass ratio (Harris, 1992). This is evident in this present study where roots generally exhibited greater percentage decrease in biomass compared to shoots when ΣPAH concentration increased from 100 mg kg^{-1} to 300 mg kg^{-1}. For instance, percentage decreases in shoot biomass from 100 mg kg^{-1} to 300 mg kg^{-1} ΣPAH were approximately 4 % and 22 % in *S. bicolor* and *M. sativa*, respectively; whereas, the root biomass similarly decreased by

233 approximately 18 % and 25 %. This finding therefore implies that the rate at which root
234 biomass proliferate may have been less compared to shoot biomass, which may have resulted
235 in the reduced root/shoot biomass ratios observed at 300 mg kg^{-1} ΣPAH compared to 100 mg
236 kg^{-1} ΣPAH. Similarly, roots are likely to be more susceptible to damage from soil contamination
237 as they are in direct contact with soil, thereby adversely affecting water and mineral transport
238 functions (Cheema *et al.*, 2010). As a result, greater energy may be expended on translocating
239 carbohydrates produced above-ground to below-ground biomass resulting in an increased
240 root/shoot biomass ratio (Harris, 1992; Reilley *et al.*, 1996). However, an evaluation of the
241 moisture content of roots and shoots after harvesting both plant species did not present any
242 significant difference ($p > 0.05$) within each of the treatments, nor between each treatment
243 and control (Figure SI 1). Hence, root functioning in terms of water transport may not have
244 been significantly impaired due to PAH-diesel oil contamination in soil during the growth
245 duration. These findings revealed reduced plant biomass and root/shoot biomass ratios for
246 both plant species in PAH-diesel oil mixture contaminated soils especially in the 300 mg kg^{-1}
247 ΣPAH treatment, however potential toxicity or stress signs were not apparent throughout the
248 growth period, which may support the notion of both plant species being tolerant of PAH-diesel
249 oil contaminated soil.

250 **3.3. ^{14}C-phenathrene mineralisation in unplanted and planted treatments**

251 The presence of a lag phase is indicative of the time needed to allow microbial adaptation
252 in soil, and it has been suggested previously that a decreasing lag phase prior to mineralisation
253 could be attributable to microbial adaptation processes (Macleod and Semple, 2002). Varying
254 lag phases were observed in the unplanted soils, which significantly shortened ($p < 0.05$) with
255 in soil-contaminant contact time (Table 3). This was more pronounced in the planted soils
256 (Table 3). Results revealed that the indigenous microorganisms in the unplanted control were
257 catabolically active. However, microbial activities were much slower as revealed by longer lag
258 phases, compared to the unplanted treatments (Table 3 and Figure 2A). The indigenous
259 microorganisms in Myerscough soil may have access to various carbon sources, including

ubiquitously-distributed PAHs, although background PAH concentrations were considered to be negligible (Adebisi, 2010).

Across all unplanted treatments, the soil spiked with 100 mg kg^{-1} ΣPAH generally exhibited shorter lag phases than those spiked with 300 mg kg^{-1} ΣPAH with and without diesel amendment at 0 d. Overall, lag phases were not significantly different within and across all unplanted treatments and these ranged from 3.84 ± 0.50 d up to 5.34 ± 0.58 d at 0 d. Only treatments with 100 mg kg^{-1} ΣPAH with and without diesel amendment presented lag phases significantly shorter ($p < 0.05$ and $p < 0.02$ respectively) when compared to untreated control soil. After 21 and 42 d, reduced lag phases, greater maximum rates and cumulative extents of mineralisation were observed in all treatments, compared to 0 d (Figures 2 - 3). Lag phases generally shortened to less than 1 d in both planted and unplanted treatments (Table 3). Rhodes *et al.*, (2008) also reported statistically shorter ($p < 0.05$) lag phases after 42 and 84 d soil-phenanthrene contact time in natural and artificial soils compared to those observed after 1 d contact time. An increase in indigenous microbial activities was observed in the planted (C3) compared to the unplanted (C4) controls (Table 3) as shown by significantly longer lag phases ($p < 0.05$) and cumulative extents of ^{14}C-phenanthrene mineralisation ($p < 0.0001$). This shows the influence of both plant species at increasing indigenous microbial activities in soil, which may have implications for contaminant biodegradation. This was further reflected by the greater CFUs of total heterotrophs and PAH degraders in the planted controls than in the unplanted control, especially for *M. sativa* (Table 3). Plant roots release root exudates containing mineralisable oxygen, water, enzymes, and a diverse array of low molecular weight carbon-containing compounds such as amino acids, sugars, organic acids, and phenolics (Bais *et al.*, 2006). These root exudates may enrich the rhizosphere and serve as readily-mineralisable carbon sources for microorganisms involved in symbiotic root-microbe interactions (Bais *et al.*, 2006; Wenzel, 2009). Continuous mineralisation and incorporation of these carbon sources increases microbial biomass, thereby supporting microbial growth, activity, and contaminant biodegradation (Guo *et al.*, 2017). Such symbiotic root-microbe interactions in soil have been previously reported for *M. sativa* (Fan *et al.*, 2008)

and *S. bicolor* (Banks *et al.*, 2003; Muratova *et al.*, 2009a). Specifically, enzymatic metabolites via cationic peroxidases from *M. sativa* and *S. bicolor* are key mechanisms for PAH biodegradation in soil in the presence of the plant species (Dubrovskaya *et al.*, 2017).

Mineralisation followed immediately after each lag phase period. At 0 d, fastest rates (0.98 ± 0.37 % $^{14}CO_2$ d^{-1}) and greatest cumulative extents of ^{14}C-phenanthrene mineralisation (59.27 ± 6.09 %) were observed only in the unplanted treatment with 100 mg kg^{-1} ΣPAH and amended with diesel ($p < 0.05$). The corresponding 300 mg kg^{-1} ΣPAH treatment exhibited the slowest rates (0.20 ± 0.002 % $^{14}CO_2$ d^{-1}) as well as the lowest cumulative extents (24.68 ± 3.48 %) of mineralisation. This trend was further reflected by a greater ratio of degraders to total heterotrophs in soil with 100 mg kg^{-1} ΣPAH, compared to soil with 300 mg kg^{-1} ΣPAH as shown in Figure SI 2A. However, microbial numbers (PAH degraders or total heterotrophs) within and across corresponding treatments were not significantly different ($p \geq 0.05$) (Table 3). In the unplanted treatments at 21 d (Table 3), rates of mineralisation were fastest ($p < 0.0001$) in soil spiked with 300 mg kg^{-1} ΣPAH especially the diesel unamended treatment; whereas, cumulative extents of mineralisation were greatest in soil with the 100 mg kg^{-1} ΣPAH without diesel. The maximum rates of mineralisation within the planted treatments in comparison to their corresponding unplanted controls were statistically similar ($p > 0.05$). This observation is consistent with previous findings where microbial respiration was not affected by plant species identity (Oyelami *et al.*, 2013), and have been suggested to be due to spatial limitations between indigenous microorganisms and plants in soil. Considering biodegradation parameters such as lag phases, fastest rates and cumulative extents of ^{14}C-phenanthrene mineralisation, observations at 0 d appeared to depict mineralisation patterns which may have been largely influenced by the concentration of freshly spiked ΣPAH in soil. It is well known that freshly spiked PAHs are more mobile and bioavailable in soil than aged PAHs (Semple *et al.*, 2007), due to minimal influence of soil-contaminant sequestration processes (Luthy *et al.*, 1997). Sorption forces are usually more apparent at lower concentrations (Pignatello and Xing, 1996), hence, soil with higher concentrations of freshly spiked PAHs may be subject to greater contaminant bioavailability compared to soil with lower concentrations (Hwang and Cutright,

316 2004b, a). Since PAHs are potentially toxic, adverse effects on soil enzymatic, as well as
317 microbial numbers and catabolic activities are likely to be observed (Kanaly and Harayama,
318 2000). In this present study, PAH inherent toxicity to indigenous microorganisms, especially
319 in soils spiked with 300 mg kg^{-1} ΣPAH, may have resulted in the pattern observed of
320 biodegradation parameters in unplanted soil at 0 d soil-PAH contact time. This result is
321 consistent with those of Couling et al. (2010) who reported greater biodegradation parameters
322 in soil spiked with lower concentrations of individual PAHs, and/or a mixture of naphthalene,
323 phenanthrene and pyrene, with single or multiple dosing of each concentrations. However, the
324 differences between biodegradation parameters at low and high PAH concentrations were
325 usually statistically similar (p > 0.05) (Couling *et al.*, 2010). In addition, while Oyelami et al.
326 (2013) observed that unplanted soils amended with different concentrations of PAH mixtures
327 showed corresponding responses in degrader numbers and activities which may have
328 resulted in consequent ^{14}C-phenanthrene mineralisation, observations from this present study
329 did not generally show such trends (Table 3 and Figure SI 2 - SI 3).
330 The rates of PAH mineralisation in planted and unplanted treatments were generally
331 statistically similar; however, cumulative extents of mineralisation also need to be considered
332 to evaluate plant-assisted biodegradation. Cumulative extents of mineralisation at 21 d were
333 significantly greater in soils spiked with 300 mg kg^{-1} ΣPAH with diesel for both *S. bicolor* (p <
334 0.0001) and *M. sativa* (p = 0.003) monocultures, compared to corresponding unplanted
335 treatments. However, a contrasting trend was generally observed (p < 0.05) in soils spiked
336 with 100 mg kg^{-1} and 300 mg kg^{-1} ΣPAH without diesel, which implied that plant-assisted
337 biodegradation in these diesel-unamended treatments was not evident in these soils. Similar
338 findings has also been reported previously (Smith *et al.*, 2011; Cennerazzo *et al.*, 2017). For
339 instance, Cennerazzo *et al*. (2017) reported that biodegradation in soil spiked with 300 mg kg^{-1}
340 phenanthrene within a 21 d *Lolium perenne* monoculture was not significantly different from
341 the unplanted treatment. In contrast, cumulative extents of mineralisation in soil spiked with
342 100 mg kg^{-1} ΣPAH with diesel from only *M. sativa* monoculture were significantly greater (p =
343 0.013) than that in corresponding unplanted treatment. Cumulative extents of mineralisation

were generally statistically similar (p > 0.05) within planted treatments (mono- and mixed cultures). The only exception was in *S. bicolor* planted soil spiked with 300 mg kg^{-1} ΣPAH and without diesel, which showed a significantly greater (p = 0.003) cumulative extents of mineralisation compared to corresponding *M. sativa* treatment. Further, cumulative extents of mineralisation within treatments were statistically similar (p > 0.05) at 42 d, except for soil spiked with 100 mg kg^{-1} ΣPAH without diesel where cumulative extents of mineralisation were significantly greater (p < 0.05) than corresponding treatment with diesel.

In this study, diesel amendment generally inhibited the rates and cumulative extents of ^{14}C-phenanthrene mineralisation in soils at 21 d and 42 d; however, the trend was not consistent, as had been previously documented for other NAPLs (Lee *et al.*, 2003). Diesel, itself being a hydrophobic non-aqueous phase liquid (Adam and Duncan, 1999), contains the greatest amount of PAHs and aromatics when compared to other medium distillate fuel oils (Wang *et al.*, 1990). It is therefore suggested that due to its hydrophobic nature, diesel may further increase PAH partitioning processes (Boyd and Sun, 1990), especially in soils with low concentrations of PAHs. Hence, decreased PAH mobility, bioavailability, toxicity, and biodegradation may occur, as also evident from the results of plant biomass and root/shoot biomass ratios previously discussed. Therefore, soil with greater PAH concentrations and amended with diesel may show greater rates and extents of mineralisation compared to one with lower PAH concentrations, especially in the presence of relevant plant species. In addition, rates and extents of mineralisation are likely to be greater in diesel unamended treatments and especially at lower PAH concentrations since an additional sorbent phase (diesel) is absent. The modifying effects of diesel amendment on rates and extents of PAH mineralisation in spiked soil may be dependent on concentration of diesel amended (Alejandra *et al.*, 2014), and these effects are likely to be greater in highly weathered field-contaminated soils (Wei *et al.*, 2017). In another study, phenanthrene degradation was reported to have increased in a pasture soil with diesel concentration of 0 - 2,000 mg kg^{-1}, but then decreased when diesel concentration was increased to 20,000 mg kg^{-1} (Swindell and Reid, 2006). Towell *et al.* (2011) also investigated the effect of cable oil concentration on biodegradation of ^{14}C-

372 phenyldodecane in an agricultural soil and reported that even though microbial respiration
373 increased with increasing oil concentration (0.001 - 10 %, w/w dry weight of soil),
374 mineralisation of ^{14}C-phenyldodecane decreased. In this present study, greater rates and
375 cumulative extents of mineralisation at 21 and 42 d were mostly observed in diesel
376 unamended treatments with similar ΣPAH concentrations (100 mg kg^{-1} or 300 mg kg^{-1}). The
377 nature of NAPLs and associated concentration are factors to be considered in PAH
378 biodegradation. Key questions to answer in future investigations are, at what concentration
379 and soil-contaminant contact time does diesel oil increase or decrease PAH biodegradation,
380 as well as identifying the mechanisms controlling the influence of diesel oil on PAH
381 bioavailability in aged soil? Such investigations may have implications for biodegradation of
382 complex PAH-diesel oil mixtures, especially in historically contaminated soils.

383 Based on previous studies (Xu *et al.*, 2006; Meng *et al.*, 2011), it was expected that a mixed
384 culture of both plant species used in this study would co-enhance rates and extents of ^{14}C-
385 phenanthrene mineralisation in soil compared to their individual monocultures, rather, the
386 mixed culture associated treatments did not significantly enhance rates and extents of ^{14}C-
387 phenanthrene mineralisation (Table 3). Either of the monocultures generally exhibited
388 significantly greater ($p < 0.05$) extents of mineralisation compared to the mixed culture.
389 Oyelami *et al.* (2013) also reported that plant species richness had no significant effects on
390 phenanthrene biodegradation in long-term aged soil. To the best of our knowledge, there have
391 been no published studies evaluating the plant-assisted biodegradation potential of *M. sativa*
392 and *S. bicolor* mixed cultures in PAH-diesel oil contaminated soil. Belowground interactions
393 between many plant roots are yet to be understood and fully investigated (Bais *et al.*, 2006).
394 Although based on daily visual assessment, plant growth aboveground in the controls did not
395 appear limited, however, plant biomass and root/shoot biomass ratios were generally more
396 reduced in mixed cultures than individual monocultures both within control and PAH-diesel oil
397 amended treatments. An antagonistic interaction between the roots of both plant species in
398 this study may not be totally excluded (Hedge and Miller, 1990; Muratova *et al.*, 2009a); this
399 is subject to further investigations. In this regard, it is speculated that greater energy may have

been expended by both plant roots towards surviving competition and associated adverse effects, rather than supporting microbial activity in the mixed culture as generally shown in Table 3. Similarly, associated microorganisms within the rhizosphere may also expend energy competing for preferable rhizospheric microhabitats rather than co-enhance biodegradation (vanVeen *et al.*, 1997). Such counter-productive survival interactions within the rhizosphere may affect the combined potential of both plant roots as well as associated microorganisms to better enhance rates and extents of ^{14}C-phenanthrene mineralisation in the PAH-diesel oil co-contaminated soil. Whether plant-assisted biodegradation of PAHs in soil, within a mixed culture of both plant species, will be observed during an extended growth period is subject to further investigations.

4. Conclusion

S. bicolor and *M. sativa* mono- and mixed- cultures were tolerant of the PAH-diesel oil amended soil. Plant-assisted biodegradation of PAH-diesel oil mixtures in soil, within the growth duration examined, was greater in *S. bicolor* or *M. sativa* monocultures compared to the mixed culture of both plant species. Overall, increase in PAH concentration reduced plant biomass and root/shoot biomass ratios, as well as adversely affected lag phases, and rates and extents of ^{14}C-phenanthrene mineralisation especially at initial stages of soil-contaminant contact. In contrast, maximum rates and cumulative extents of ^{14}C-phenanthrene mineralisation were greater at advanced stages of soil-contaminant contact time especially in the more concentrated PAH-contaminated soils with monocultures of the plant species used. Diesel amendment supported plant biomass production as well as increase in root/shoot biomass ratios, however, appeared to inhibit rates and extents of ^{14}C-phenanthrene mineralisation in soil. The mechanisms through which diesel oil controls the fate and behaviour of complex PAH mixtures in soil should be further investigated. These may have implications for the risk assessment and remediation of PAHs in soil.

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6. Supplementary Information

Supplementary data on “Mineralisation of ^{14}C-phenanthrene in PAH-diesel contaminated soil: Impact of *Sorghum bicolor* and *Medicago sativa* mono- or mixed culture” are available.

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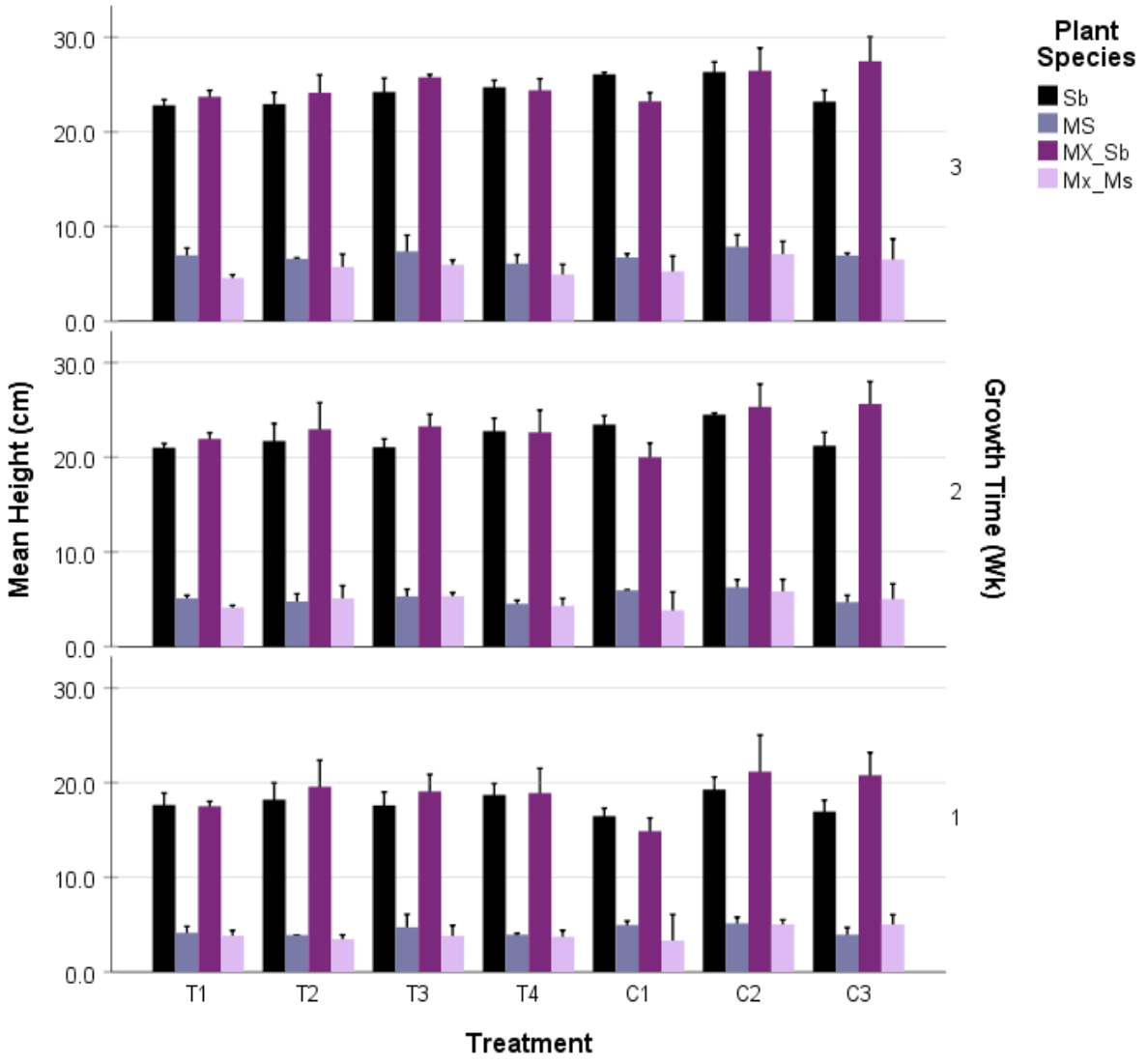
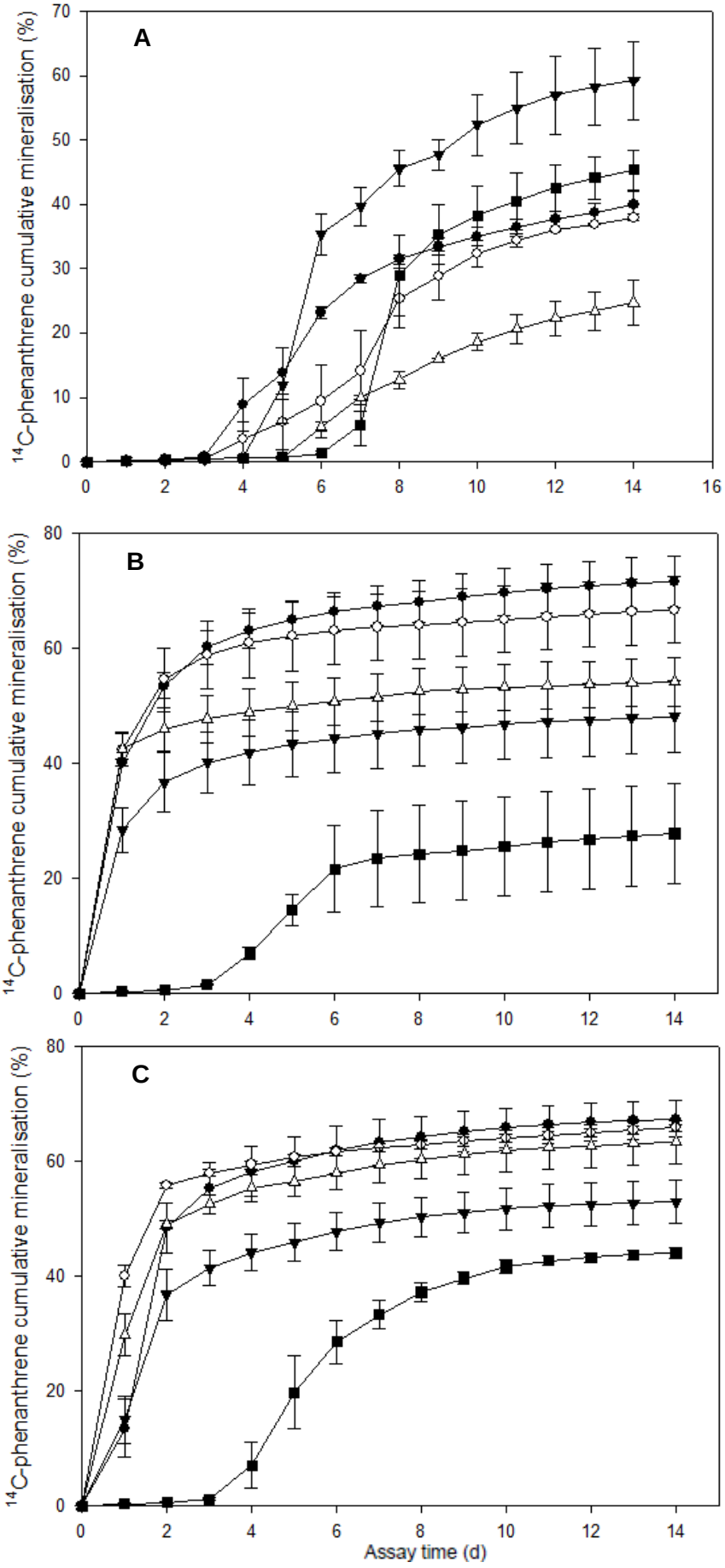
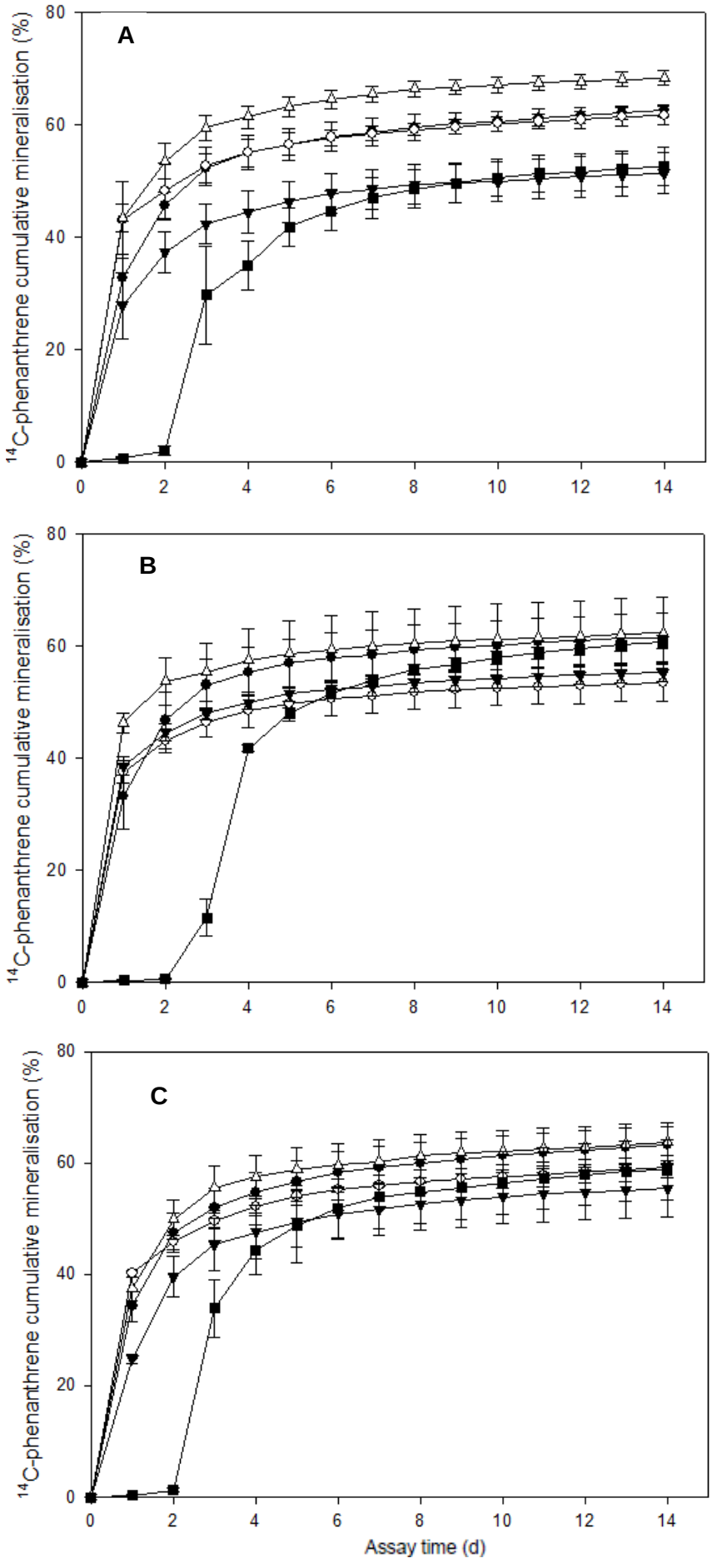
Figure 1. Weekly plant heights across all treatments ($p > 0.05$).

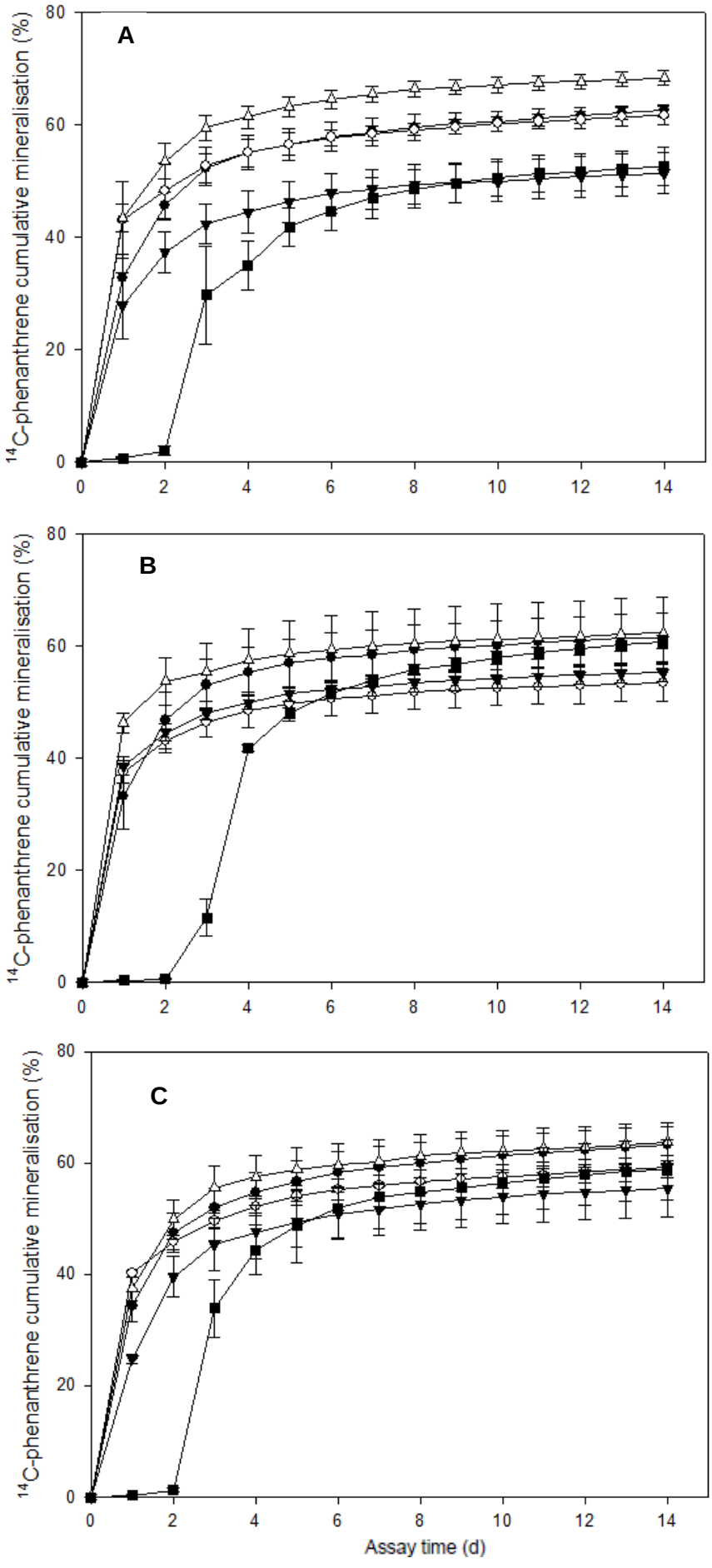
Figure 2. Development of ^{14}C-phenanthrene mineralisation at 0 d (A), 21 d (B) and 42 d (C) respectively. Control, C4 (■); C5 (▼), and C6 (Δ); C7 (●); C8 (○). Note the different scale on y-axis.

Figure 3. Development of ^{14}C-phenanthrene mineralisation at 21 d in monocultures of *S. bicolor* (A) and *M. sativa* (B), and mixed culture (C) respectively. Control = C3 (■); T1 (▼); T2 (Δ); T3 (●); T4 (○). Note the different scale on y-axis.


Plant Species
Sb
MS
MX_Sb
Mx_Ms
Mean Height (cm)
Growth Time (Wk)
30.0
20.0
10.0
0.0
3
2
1
T1
T2
T3
T4
C1
C2
C3
Treatment


A
B
C
^{14}C-phenanthrene cumulative mineralisation (%)
Assay time (d)


A
B
C
^{14}C-phenanthrene cumulative mineralisation (%)
Assay time (d)
0
20
40
60
80
0
2
4
6
8
10
12
14


A
B
C
^{14}C-phenanthrene cumulative mineralisation (%)
Assay time (d)
0
20
40
60
80
0
2
4
6
8
10
12
14